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14. ABSTRACT

Apoptosis is a program of cellular suicide that removes individual cells from the midst of a living tissue without destroying overall tissue architecture. In response to chemotherapeutic agents, cells die by apoptosis. Moreover, inhibition of apoptosis is a hallmark of cancers. In this proposal, we proposed to understand the molecular basis for apoptosis induced by the pro-apoptotic protein, Reaper. Having purified and characterized a protein, Scythe, acting downstream of Reaper to trigger mitochondrial cytochrome c release and cell death, we wished to determine how these proteins might cooperate to execute the apoptotic program..

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None provided.

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Reaper is a central regulator of cell death in the fly, Drosophila Melanogaster. In characterizing the action of Reaper protein, we found that Reaper expression in human breast cancer cells could promote apoptotic cell death. We proposed to explore the mechanistic basis for this cell death, focusing on the action of a protein, Scythe, which we purified as a Reaper-interacting protein. In addition, during the course of this work, we became interested other features of Reaper action, including its ability to antagonize Inhibitors of Apoptosis (IAP proteins), thereby enhancing cell death. This work resulted in number of publications from both the original holder of this grant (Michael Olson) and from the student who assumed the funding after Dr. Olson's completion of the MD/PhD degree.

Task s from Statement of Work: 1)To identify the *Scythe*-interacting protein (SCF) responsible for inducing cytochrome c release from mitochondria, 2) to clone and 3) characterize SCF and 4) to examine its therapeutic potential.

As described in a previous progress report, we found that Scythe interacted directly with Hsp70 to control protein folding of associated factors. We found subsequently that reconstitution of mitochondria with purified Scythe, Hsp70, the Hsp70 auxilliary factor, Hdj-1 and Reaper could result in mitochondrial cytochrome c release (leading to caspase activation). This suggested that a mitochondrial factor(s) might be modulated by Hsp70-mediated protein folding. We have now found that components of the mitochondrial fission/fusion machinery likely to be associated with mitochondria (and previously reported to regulate cytochrome c release) are modulated by Reaper/Scythe. This work is ongoing, but the key finding is that Scythe knock-out cells possess fragmented mitochondria and that this phenotype is also seen upon Reaper expression. Furthermore, we have found that the regulator of mitochondrial fusion, Drp-1 changes in its oligomerization state upon Reaper expression. We are currently examining the possibility that Scythe-Hsp70 promotes Drp-1 oligomerization in response to Reaper.

In the course of performing this work, we made many fundamental discoveries concerning Reaper action. We found that part of Reaper's pro-apoptotic activity came from its ability to promote auto-ubiquitination and degradation of the IAP (inhibitor of apoptosis) proteins. Interestingly, we found that this function of Reaper relied upon its ability to direct IAP proteins to the mitochondria. We recently found that a specific region of Reaper (the GH3 domain) is responsible for this mitochondrial localization and that this results from GH3-lipid interactions at the mitochondrial outer membrane.

In a twist on this, we found that IAP proteins could also eliminate Reaper protein by ubiquitinating it and targeting it for proteosomal degradation. Thus, IAPs can antagonize Reaper function as well as the converse. It is not yet clear how the Scythe regulatory pathways described in our original proposal and the IAP pathways that we came upon in the course of this work intersect. In exciting preliminary data, we have found that Scythe overexpression dampens and Scythe ablation through RNAi enhances Reaper-mediated IAP degradation.

In collaboration with the Okada and Mak laboratories, we have more recently seen that human Scythe is required for p300-mediated acetylation of p53. This is obviously of

potential importance in the regulation of p53-mediated cell death in breast cancer. It is also possible that p53 is a component of the Scythe-regulated cytochrome c-releasing factor in that p53 has recently been shown to act directly at the mitochondria to trigger cytochrome c release and cell death.

Key research accomplishments:

- Demonstration that Scythe-Hsp70 interactions are key to Reaper-mediate cytochrome c release and induction of apoptosis in vertebrate cells
- Demonstration that Reaper promotes the auto-ubiquitination and destructin of IAP proteins
- Determination that IAP proteins can "fight back" by promoting Reaper ubiquitination and degradation
- Demonstration that Reaper associates with mitochondria via its GH3 domain and that this association is required for Reaper-mediated IAP degradation
- In collaboration with Okada lab, demonstration that p300-mediated acetylation of p53 is regulated by Scythe

Reportable outcomes: (note that this project was undertaken by two different students as the first PI graduated and the award was reassigned. Hence, publications by both students, Michael Olson and Eugene Gan are listed below).

Olson, M. and Kornbluth, S. (2001). Mitochondria in apoptosis and human disease. *Curr. Moec. Medicine*. **1**: 91-122.

Tashker, J.S., M. Olson, and S. Kornbluth (2002). Post-cytochrome c protection from apoptosis conferred by a MAPK pathway in Xenopus egg extracts. *Mol Biol Cell **13**:* 393-401.

Holley, C., M. Olson, D. Colon-Ramos, and S. Kornbluth (2002). Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nature Cell Biol,* **4**: 439-444.

Olson, M.R., Holley, C., Yoo, S.J. Huh, J.R. , Hay, B.A. and Kornbluth, S. (2003). Reaper is regulated by IAP- mediated ubiquitination. *J. Biol. Chem***, 278***: 4028-34.*

Olson, M.R., Holley, C., Gan, E.C., Colon-Ramos, D.A., Kaplan, B., and Kornbluth, S. (2003). A GH3- like domain in Reaper required for mitochondrial localization and induction of IAP degradation. *J Biol. Chem,* **278**: 44758-44768

Colon-Ramos, D.A., P.M Irusta, E.C. Gan, M.R. Olson, J. Song, R.I. Morimoto, R.M. Elliott, M. Lombard, R. Hollingsworth, J.M. Hardwick, G.K. Smith, and S. Kornbluth. (2003). Inhibition of translation and induction of apoptosis by Bunyaviral non-structural proteins bearing sequence similarity to Reaper. *Mol. Biol. Cell,* **14**: 4162-4172.

Colon-Ramos, D.A., C. Shenvy, D.H. Weitzel, E.C.Gan, R. Matts, J. Cate, and S. Kornbluth. (2006). Direct Ribosomal binding by a cellular inhibitor of translation. *Nature Structural and Molecular Biol*. **13**: 103-111.

Sasaki, T., E. Gan, A. Wakeham, S. Kornbluth, T.W. Mak, and H. Okada. (2007). HLA-B-associated transcript 3 (Bat3)/Scythe is essential for p300-mediated acetylation of p53. *Genes and Devel., in press*

Conclusion: This study was designed to understand the mechanism of action of Reaper, a key apoptotic regulator. Although we made some good progress on elucidating the mechanism of action of the Reaper-interacting protein Scythe (the focus of the proposal), we made more substantial progress in understanding the effects of Reaper on IAP proteins. This is of particular interest as IAP antagonists (ie. Reaper-mimetics) are being evaluated by a number of pharmaceutical companies as cancer therapeutics.